Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 11634–11639, October 1996
Biophysics

Transbilayer inhibition of protein kinase C by the lipophosphoglycan from *Leishmania donovani*

(sucrose-loaded vesicles/membrane partitioning/protein kinase C activation/enzyme kinetics)

JENNIFER R. GIORGIONE*, SALVATORE J. TURCO†, AND RICHARD M. EPAND*‡

*Department of Biochemistry, McMaster University, Health Sciences Centre, 1200 Main Street West, Hamilton, ON Canada, L8N 3Z5; and †Department of Biochemistry, University of Kentucky College of Medicine, Lexington, KY 40536

Communicated by Tibor Farkas, Hungarian Academy of Sciences, Szeged, Hungary, August 1, 1996 (received for review January 2, 1996)

ABSTRACT **Lipophosphoglycan (LPG), the predominant molecule on the surface of the parasite *Leishmania donovani*, has previously been shown to be a potent inhibitor of protein kinase C (PKC) isolated from rat brain. The mechanism by which LPG inhibits PKC was further investigated in this study. LPG was found to inhibit the PKC_α-catalyzed phosphorylation of histone in assays using large unilamellar vesicles composed of 1-palmitoyl, 2-oleoyl phosphatidylserine and 1-palmitoyl, 2-oleoyl phosphatidylcholine either with or without 1% 1,2 diolein added. The results also indicated that while PKC binding to sucrose-loaded vesicles was not substantially reduced in the presence of LPG at concentrations of 1–2%, the activity of membrane-bound PKC was inhibited by 70%. This inhibition of the membrane-bound form of PKC is not a consequence of reduced substrate availability to the membrane. However, K_m shifted from ≈31 ± 4 μM to 105 ± 26 μM in the presence of 5% LPG. LPG caused PKC to bind to membranes without inducing a conformational change as revealed by the lack of an increased susceptibility to trypsin. An LPG fragment containing only one repeating disaccharide unit was not as effective as the entire LPG molecule or of larger fragments in inhibiting the membrane-bound form of the enzyme. The shorter fragments were also less potent in raising the bilayer to hexagonal phase transition temperature of a model membrane. LPG is also able to inhibit the membrane-bound form of PKC_α from the inner monolayer of large unilamellar vesicles, the opposite monolayer to which the enzyme binds in our assay. Inhibition is likely a result of alterations in the physical properties of the membrane. To our knowledge, this is the first example of a membrane additive that can inhibit the membrane-bound form of PKC in the presence of other lipid cofactors.**

Lipophosphoglycan (LPG) is the major glycoconjugate found on the surface of the promastigote form of *Leishmania donovani*, a protozoan parasite that causes human leishmaniasis (reviewed in refs. 1 and 2). The structure of the *L. donovani* LPG consists of an average of 16 repeating units of Gal(β1,4)Manα1-PO_4 units linked to a glycan core of 3 Gal, 2 Man, 1 Glc-PO_4, and one unacetylated glucosamine residue. The entire polysaccharide portion of LPG is anchored by a 1-*O*-alkyl-2-lyso-phosphatidylinositol, resulting in a highly negatively charged molecule of average M_r of 9 kDa. LPG is expressed on all species of *Leishmania* with conservation of the lipid anchor and glycan core and with some variation in the repeating units. A number of interesting functions have been proposed for LPG (1), including protection of the parasite against microbicidal activities in *Leishmania*–macrophage interactions. Macrophages undergo an oxidative burst during phagocytosis, which leads to the production of oxygen radicals. Protein kinase C (PKC), an enzyme involved in signal transduction, is thought to be a mediator of the oxidative burst (3) and other cellular processes. PKC acts by phosphorylating serine and threonine residues on its protein substrates. The classical isoform of PKC used in this study, is regulated by calcium, phosphatidylserine (PS), and diacylglycerol.

In previous studies, purified LPG was shown to be an inhibitor of PKC activity *in vitro* as well as an inhibitor of PKC-mediated events in living phagocytic cells (4–9). Thus, it appears that an effective inhibition of PKC activity achieved by LPG may represent a critical step for successful establishment of *Leishmania* promastigotes within host macrophages. In this study, we undertook an investigation to better understand the relationship between LPG and inhibition of PKC activity. Using PKC purified from rat brain, as well as PKC_α purified from a bacculovirus expression system, we have found that LPG inhibits the membrane-bound form of PKC.

In vivo, LPG and PKC are found on opposite sides of the membrane when a *Leishmania* parasite approaches a host cell. LPG is intercalated on the macrophage cell surface during phagocytosis of the parasite into the macrophage, as observed by immunofluorescence using anti-LPG monoclonal antibodies (10), while PKC binds to the cytoplasmic side of the plasma membrane. We demonstrate that despite the positions of LPG and PKC on opposing monolayers, inhibition is still observed. This demonstrates that inhibition of PKC by LPG may be of biological importance in the initial stages of infection by *Leishmania* and that this inhibition must be a consequence, at least in part, of effects of LPG on membrane physical properties that can be transmitted across the bilayer.

EXPERIMENTAL PROCEDURES

Materials. Lipids were purchased from Avanti Polar Lipids. Histone H-I was from GIBCO/BRL. BSA fraction V, protamine sulfate, ATP sodium salt, and EGTA were from Sigma. [γ-^{32}P]ATP and [9,10-^{3}H] dipalmitoyl phosphatidylcholine (PC) were from NEN. The peptide acetyl-Phe-Lys-Lys-Ser-Phe-Lys-Leu-amide was purchased from National Research Council (Canada). The peptide Arg-Arg-Arg-Arg-Tyr-Gly-Ser-Arg-Arg-Arg-Arg-Arg-Arg-Tyr (R-peptide) was synthesized at the peptide facility of the University of Kentucky (Lexington). LPG was isolated and purified from *L. donovani* as described (11).

LPG Fragments. LPG was cleaved at acid-labile phosphodiester bridges (12). Purified LPG was treated with 0.02 M HCl at 60°C for 1 min. The sample was then neutralized with NaOH

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: LPG, lipophosphoglycan; PKC, protein kinase C; SLV, sucrose-loaded vesicle; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; R-peptide, Arg-Arg-Arg-Arg-Tyr-Gly-Ser-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Tyr; DG, 1,2-diolein; T_H, bilayer to hexagonal phase transition temperature; PS, phosphatidylserine; PC, phosphatidylcholine, POPS, 1-palmitoyl,2-oleoyl PS; POPC, 1-palmitoyl, 2-oleoyl PC; DEPE, dielaidoyl phosphatidylethanolamine.

‡To whom reprint requests should be addressed. e-mail: Epand@fhs.csu.McMaster.ca.

and subjected to gel filtration chromatography on Sephadex G150 (1 × 100 cm) equilibrated with 0.04 N NH_4OH and 1 mM EDTA; 0.6 ml fractions were collected and aliquots were assayed for carbohydrate by phenol-surfuric acid (13). Three pools were taken based on relative sizes due to partial depolymerization of the repeating units. The pools were desalted by dialysis. The resulting LPG fragments contained an average of 1, 6, or 12 repeating Gal(β1,4)Manα1-PO_4 units.

PKC Purification. Rat brain PKC was purified by a modification of the procedure of Huang *et al.* (14), as described elsewhere (15). Purified PKC displayed a single band on the silver-stained electrophoresis gel. The specific activity of the enzyme for histone in a micellar assay (16) was 1–2 mmol·mg·min. The phospholipid independent activity in this assay did not exceed 2% of the total kinase activity. Some experiments were performed with rat brain PKC purchased from Molecular Probes.

PKC_α was purified from insect cells overexpressing the α isoform in the bacculovirus system. Cells were a gift from David Burns and Nancy Rankl (Sphinx Pharmaceutical, Durham, NC). The procedure was a modification of that of Stabel *et al.* (17). Briefly, insect cell pellets were lysed in 2 mM benzamidine/1% Triton X-100/50 mM Tris·HCl, pH 7.5/2 mM EDTA/10 mM EGTA/1 mM dithiothreitol/40 μg/ml leupeptin/200 μM phenylmethylsulfonyl fluoride. The mixture was homogenized and then centrifuged at 200,000 × *g* for 50 min. The supernatant was applied to a Q-Sepharose column (Pharmacia; 2.5 cm × 20 cm) equilibrated with DEAE buffer [1 mM EDTA/1 mM EGTA/20 mM Tris·HCl, pH 7.5/10% (vol/vol) glycerol]. DEAE buffer alone, then DEAE containing 0.05 M, 0.15 M, 0.45 M, and 1.0 M KCl were used for elution. PKC_α was eluted with the 0.15 M KCl between fractions 20–30. Collected fractions (3.5 ml) were assayed for PKC activity as for the rat brain PKC (16). Fractions with the highest activity were pooled, adjusted to 1.5 M KCl and loaded onto a phenyl-Sepharose column (Pharmacia; 1.0 cm × 10 cm) equilibrated with 1.5 M KCl/1 mM EDTA/1 mM EGTA/20 mM Tris·HCl, pH 7.5/10% glycerol. PKC_α was eluted with a 100-ml salt gradient from 1.5 to 0 M KCl. Fractions (2 ml) were tested for activity, pooled, and concentrated to ≈0.4 mg/ml by ultrafiltration using an Amicon YM30 membrane and stored at −70°C in 50% glycerol. The specific activity of PKC_α was similar to that of rat brain PKC.

Phospholipid Vesicles. Lipid films were made by dissolving phospholipids in 2:1 (vol/vol) chloroform:methanol, and drying under a stream of nitrogen, followed by desiccation under vacuum for 2 hr. Films were suspended in either a buffer of 5 mM $MgCl_2$, 20 mM Tris·HCl, 100 mM KCl, or a sucrose buffer of 0.17 M sucrose in 5 mM $MgCl_2$ and 20 mM Tris·HCl, pH 7.0. Vesicles were subjected to five freeze-thaw cycles and extruded through two 0.1 μm polycarbonate filters in a microextruder. Vesicles containing sucrose were suspended in the $MgCl_2$·Tris·KCl buffer and centrifuged at 100,000 × *g* for 30 min to dilute out the sucrose.

LPG Vesicles. *Incorporation of LPG into the outer monolayer only.* An aqueous solution containing purified LPG or LPG fragments was incubated with LUVs (large unilamellar vesicles) or SLVs (sucrose-loaded vesicles) for 20 min in a buffer of 100 mM KCl/5 mM $MgCl_2$/20 mM Tris·HCl, pH 7.0. The mol % of LPG is always given with respect to the total lipid present, regardless of the sidedness of LPG. Measurement of LPG incorporation into these vesicles using SLVs and LPG that was biosynthesized using ^{3}H-mannose, showed that these conditions resulted in essentially quantitative incorporation of LPG into the vesicles.

Incorporation of LPG into inner and outer monolayers. An aqueous solution of LPG was added to dried lipid films before the five freeze-thaw cycles. These multilamellar vesicles were then extruded to form LUVs or SLVs.

Removal of LPG from outer monolayer. LUVs were prepared with LPG on both inner and outer monolayer, or on the outer monolayer only. LUVs were incubated with a 5-fold excess of sucrose-loaded multilamellar vesicles (MLVs) for 48 hr. During this time, LPG was exchanged from the outer monolayer of the LUVs to the MLVs. The MLVs were sedimented at 12,000 × *g* for 10 min. The supernatant containing the LUVs with no LPG, or LPG on the inner monolayer only were used in the PKC activity assays. The MLVs consisted of 1-palmitoyl,2-oleoyl PC (POPC) only, and had no effect on the assay. The exchange of LPG was monitored by incorporating a small amount of tritium labeled LPG into the vesicles.

PKC Binding Assays. The SLV assay was modified from the procedure of Rebecchi *et al.* (18) as described elsewhere (15). Briefly, PKC was incubated with SLVs and then centrifuged at 100,000 × *g* for 30 min at 25°C to separate the membrane-bound enzyme. The pellet and supernatant were assayed under identical conditions for enzyme activity toward the R-peptide. The substrate used was one which was not sensitive to the presence or absence of LPG. However, an equal amount of vesicles were added to the supernatant fraction prior to the activity assay so that the measured activity in supernatant and pellet fractions would be directly comparable. The buffer contained 100 mM KCl/5 mM $MgCl_2$/20 mM Tris·HCl, pH 7.0/200 μM $CaCl_2$/0.3 mg/ml BSA.

PKC Activity Assays. The activity assay toward histone, protamine sulfate, or synthetic peptide was performed as previously described (15, 19). The buffer was the same as that used for the binding assays. Histone or protamine sulfate was added to a final concentration of 0.2 mg/ml (in 250 μl total volume), acetyl-FKKSFKL-amide was used at a final concentration of 90 μM. Phospholipid was 85 μM in the form of LUVs, [γ-^{32}P]ATP [0.2 mCi/ml (1 Ci = 37 GBq)] was 20 μM, and PKC was 100 ng/ml.

Differential Scanning Calorimetry. Bilayer to hexagonal phase transition temperatures were measured as described previously (20). Briefly, lipid films of dielaidoyl phosphatidylethanolamine (DEPE) were suspended in buffer to a final total lipid concentration of 10 mg/ml, and LPG fragments were added. An MC-2 high sensitivity calorimeter was used (Microcal, Amherst, MA), with a heating scan rate of 41 K/hr.

Determination of K_m and V_{max}. Activity assays were performed using 100 μM lipid [70:29:1 PS:PC:1,2-diolein (DG) ± 5% LPG] with the MARCKS peptide as a substrate. Other conditions are the same as described above. K_m and V_{max} were determined using the program GRAFIT 3.0 (Erithacus Software, Staines Middlesex, U.K.).

Trypsin Cleavage of PKC_α. The procedure was a modification of that of Newton and Koshland (21). PKC_α (300 ng) was incubated with 200 μM Ca^{2+}, 400 μM LUVs of 70:29:1 PS:PC:DG ± 5% LPG at 25°C. Ten microliters of 88 units/ml trypsin was added, and the mixture was incubated for 15 min at 30°C. Reaction products were separated by SDS/PAGE on a 7% acrylamide gel that was then silver stained.

RESULTS

LUVs consisting of 50 mol% 1-palmitoyl, 2-oleoyl PS (POPS), 0.5–4.0 mol% LPG, and the remainder POPC, inhibited PKC-catalyzed phosphorylation of histone relative to LUVs composed of 1:1 POPS/POPC (Fig. 1*a*). Similar results showing a dose-dependent LPG inhibition of PKC activity using Triton micelles containing PS and DG have previously been reported (9). The largest decrease in activity is seen between 0 and 0.5% LPG.

To ascertain whether the inhibition of PKC activity was caused by LPG interfering with binding of the enzyme to the membrane or by inhibiting the membrane-bound form of PKC, a modified form of the SLV binding assay for PKC was used. The original method (15) used protamine sulfate as a substrate

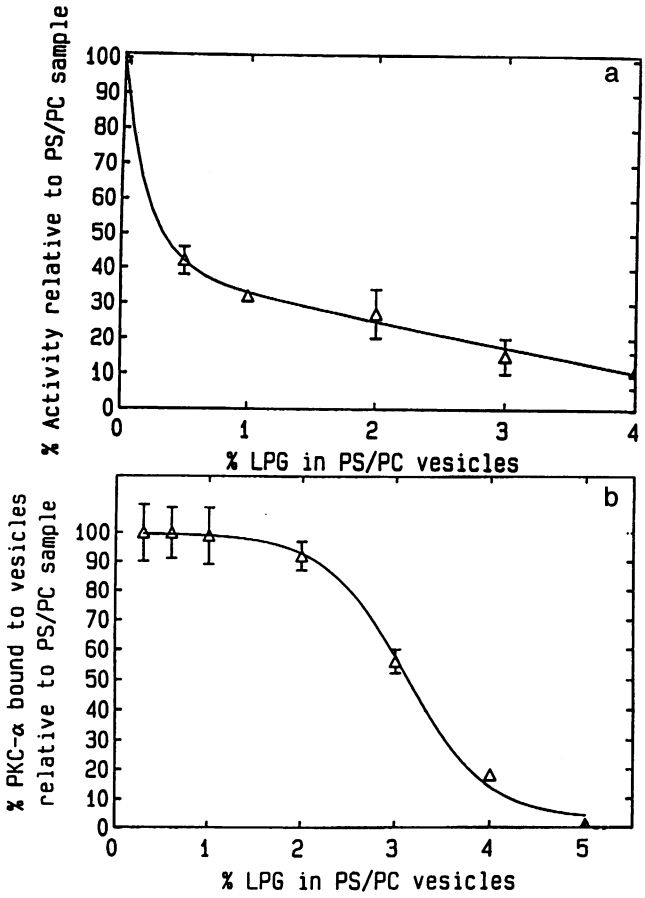

FIG. 1. LPG inhibition of PKC-catalyzed phosphorylation of histone and binding of PKC$_\alpha$ to SLVs. The LUVs were composed of 50 mol% POPS, 0.3%-5.0% LPG, and the remainder POPC. (*a*) Activity. The activity is expressed as a percentage of the activity obtained using LUVs without LPG that was 245 pmol per min per mg. (*b*) Binding. The binding is expressed is a percentage of the binding obtained with vesicles without LPG that was 87% of the added enzyme. Data are expressed as the mean of triplicate determinations ± SD at each concentration of LPG.

for PKC to determine the distribution of the enzyme between membrane and soluble fractions. However, PKC-catalyzed phosphorylation of protamine sulfate was found to be activated by LPG thus complicating the analysis. Using LUVs of 50 mol% POPS with the remainder POPC, 1–3% LPG increased phosphorylation of protamine sulfate up to 50% (not shown). A synthetic peptide, the R-peptide, has a sequence corresponding to a segment of protamine. Like protamine sulfate, it is phosphorylated by PKC in a manner largely independent of Ca^{2+} or lipid (22). The rate of phosphorylation of the R-peptide is not affected by the presence of vesicles containing 1–5% LPG (data not shown). This substrate was therefore used instead of protamine sulfate in the PKC binding assay. The results showed that 1 or 2% LPG modestly inhibited the binding of PKC$_\alpha$ to membranes but that 5% LPG completely prevented binding (Fig. 1*b*). The results obtained using rat brain PKC (data not shown) were similar to those shown using PKC$_\alpha$. Therefore, comparisons between experiments performed using the two different PKC preparations are valid, in addition to the fact that both preparations are essentially composed of conventional PKC isoforms.

The percentage of PKC bound to vesicles (Fig. 1*b*) with a particular LPG content, is higher than the relative activity of PKC toward histone (Fig. 1*a*) for the same vesicle composition. DG is known to increase the binding affinity of PKC for PS-containing membranes (15). Therefore, adding 1% DG and increasing the percentage of PS of the vesicles to 70% resulted in nearly 100% of the PKC binding to LUVs (using the R-peptide as substrate) even in the presence of 5% LPG (Fig. 2*a*). Since it is possible to achieve ≈100% binding in the presence of LPG (at 70 mol% PS), LPG's effects on PKC's binding to membranes is not likely due to a steric hindrance caused by the bulkiness of the molecule, but possibly by an effect on the physical properties of the membrane. PKC phosphorylation of histone is reduced by LPG even at high mol% PS where all of the enzyme is bound to the membrane (Fig. 2*b*).

It was important to determine whether inhibition of PKC phosphorylation of histone by LPG was due to reduction in the amount of substrate available at the membrane. It has been shown that the peptide substrate acetyl-FKKSFKL-amide (MARCKS peptide) attains its maximal rate of phosphorylation at lower mol fractions of PS than is the case for histone (15). LUVs with 70% POPS and 1% DG should support a maximum rate of PKC catalyzed phosphorylation of this heptapeptide. The fact that LPG caused a similar inhibition of the phosphorylation of this substrate (data not shown) as it did for histone indicates that LPG is inhibiting the membrane-bound form of PKC and is not acting by reducing substrate binding to the lipid bilayer.

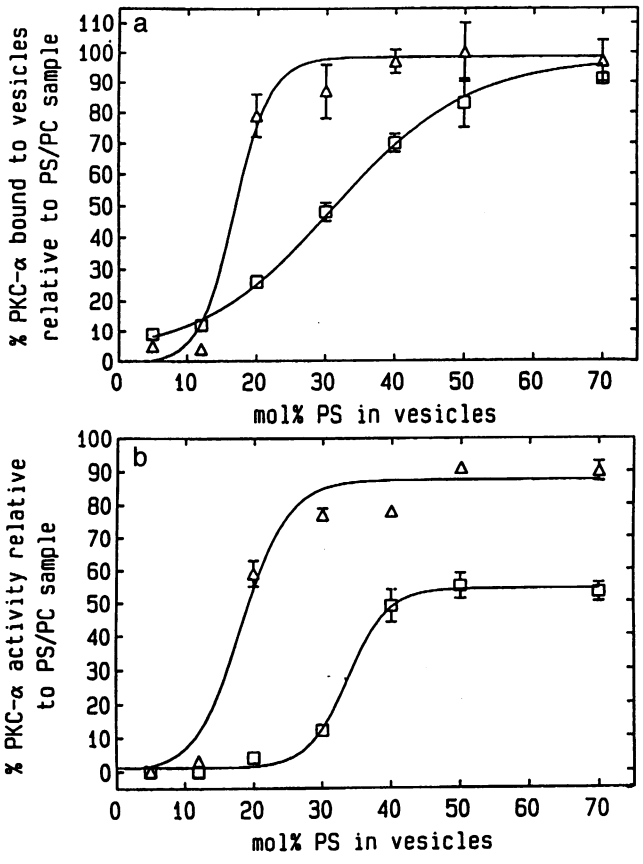

FIG. 2. Binding of PKC$_\alpha$ to SLVs and PKC-catalyzed phosphorylation of histone. The vesicles were composed of the indicated amount of POPS, 1 mol% DG, and either 2% LPG (△) or 5% LPG (□) with the remainder being POPC. (*a*) Binding. The binding is expressed as a percentage of that obtained with vesicles containing no LPG. This corresponds to ≈100% of the added enzyme bound for vesicles containing 20–70 mol% PS, 84% and 21% for vesicles containing 12 and 5 mol% PS, respectively. (*b*) Activity. The activity is expressed as a percentage of that obtained with vesicles containing no LPG. This corresponds to a specific activity of 3.8, 4.1, 4.5, 2.6, 2.1, 1.9, and 0.65 nmol·min·mg for vesicles containing 70, 50, 40, 30, 20, 12, and 5 mol% PS respectively. Data is expressed as the mean of triplicate determinations ± SD for each concentration of POPS.

Since LPG is negatively charged it would be expected to increase the accumulation of cationic substrates at the membrane interface by the Gouy–Chapman electrical double layer effect. Thus, if LPG had any effect on PKC activity through changes in substrate accumulation at the membrane, it would be expected to increase the activity of the enzyme. SLVs were incubated with the MARCKS peptide and centrifuged as in the binding assay to separate free from membrane-bound MARCKS peptide. This showed that there were similar amounts of peptide bound to LUVs composed of 70:29:1 PS:PC:DG either with or without 5% LPG (data not shown). Thus, under the conditions used, one would not anticipate any change in substrate availability to the enzyme.

K_m and V_{max} were determined for the MARCKS peptide. At a lipid concentration of 100 μM, V_{max} was 0.034 ± 0.001 pmol phosphate/min without LPG, and 0.034 ± 0.003 with 5% LPG with vesicles of 70:29:1 PS:PC:DG. K_m was 31 ± 4 μM in the absence of LPG, and 105 ± 26 μM with 5% LPG (Fig. 3).

PKC_α was subjected to cleavage by trypsin, in the presence of 70:29:1 PS:PC:DG vesicles, with and without added 5% LPG. Binding to membranes results in a conformational change in PKC leading to the exposure of the enzyme's hinge region, which contains a trypsin cleavage site (21). The silver-stained SDS/PAGE gel (Fig. 4) revealed that PKC_α was much less sensitive to cleavage when bound to vesicles containing LPG (lane 2) than when bound to those lacking LPG (lane 1). In the presence of LPG, most of the PKC_α remains uncleaved, while in its absence most of the PKC_α is cleaved to a 45-kDa catalytic and 35-kDa regulatory domain. Note that the amount of intact PKC_α remaining in the presence of LPG (lane 2) is similar to the amount obtained when no vesicles are present (lane 3), although the binding assays reveal that under these conditions, nearly 100% of the PKC_α is membrane-bound. Therefore, PKC must be bound in a nonactivated conformation, similar to that which occurs in solution and which does not expose the hinge region. It is evident that PKC's regulatory domain interacts differently with an LPG containing membrane, as suggested by the smear in lane 2 instead of the 35-kDa band that appears in lane 1. This is likely, since LPG was found to have no effect on the activity of protein kinase M (the catalytic fragment of PKC) in an *in vitro* assay (9). LPG-containing vesicles were not found to affect the activity of trypsin, in assays using either histone or casein as substrates, relative to vesicles without LPG (data not shown).

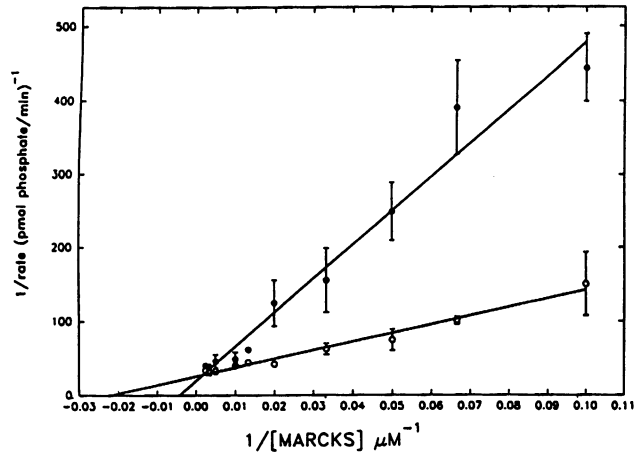

FIG. 3. Lineweaver–Burke plot for the phosphorylation of the MARCKs peptide by PKC_α. LUVs were composed of 70 mol% POPS, 1 mol% DG, and the remainder POPC, either without (○) or with 5% LPG (●). Actual K_m and V_{max} values were calculated using GRAFIT 3.0. Data are expressed as the mean of triplicate determinations of at least two separate experiments.

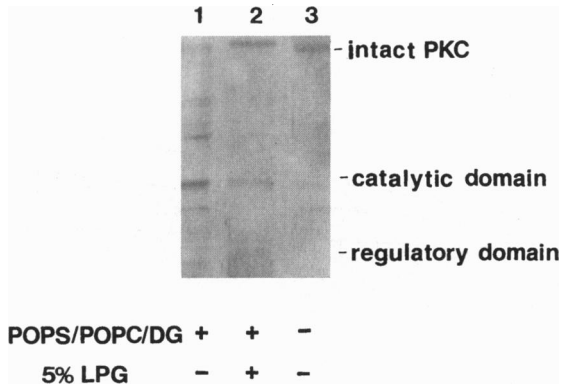

FIG. 4. Silver-stained polyacrylamide gel showing PKC_α's sensitivity to tryptic hydrolysis. PKC_α (300 ng) was incubated with LUVs composed of 70 mol% PS, 1 mol% DG, and the remainder POPC, either without (lane 1) or with 5% LPG (lane 2), then treated with trypsin as described in *Experimental Procedures*. Lane 3 was in the absence of LUVs.

To discover that portion of LPG is responsible for the inhibition of the membrane-bound form of PKC, we used LPG fragments consisting of an average of 1, 6, or 12 repeating Gal(β1,4)Manα1-PO_4 units in the binding and activity assays in the presence of 1 mol% DG. It was found that LPG fragments containing an average of 12 and 6 repeating disaccharide units inhibited the membrane-bound form of PKC to a similar extent as the full length molecule of an average of 16 repeating units. However, there was much less of an effect on membrane-bound PKC by LPG fragments containing an average of only one disaccharide unit (data not shown). In contrast to previous findings using Triton micelles (8), the 1-*O*-alkylglycerol portion of LPG (the lipid core containing no carbohydrate) was found to be ineffective in inhibiting the activity of PKC in our LUV assay system (data not shown), or at raising the bilayer to hexagonal phase transition temperature (T_H)[§] of DEPE (data not shown). Therefore, both enzyme inhibitory effects as well as effects on lipid polymorphism were lost when most of the carbohydrate portion of LPG was removed.

The inhibitory effect of LPG on PKC activity may be a consequence of its bilayer stabilizing effects on membranes. We investigated if the loss of inhibitory activity of the smaller LPG fragment could be correlated with its reduced effectiveness in raising the T_H of a synthetic phospholipid. DEPE is a model system often used to measure changes in T_H. The changes in T_H of DEPE reflect the effects of the additive on membrane intrinsic curvature. These shifts in T_H have been correlated with changes in PKC activity (23). Indeed there was a progressive loss in the bilayer stabilizing ability of the LPG fragments as the size of the fragments became smaller (Fig. 5). This is consistent with the suggestion that the bilayer stabilization of membranes by LPG is responsible for its inhibitory action, but steric factors may also play a role.

Strong evidence for the involvement of membrane physical properties is given by the finding that LPG is able to inhibit both the activity and binding of PKC_α even from the opposite monolayer to which the enzyme binds. LUVs consisting of 30:69:1 PS:PC:DG + 2.5% LPG on each side of the membrane inhibited PKC_α catalyzed phosphorylation of histone as well as its binding to vesicles to a greater extent than did the same LUVs containing 2.5% LPG on the outer monolayer only (Fig. 6). LPG is inhibiting the membrane-bound form of PKC_α,

[§]T_H is the temperature at which model bilayers form the inverted hexagonal phase. This phase forms when the bilayer is destabilized by increasing the intrinsic curvature of each monolayer (see ref. 23 for further explanation).

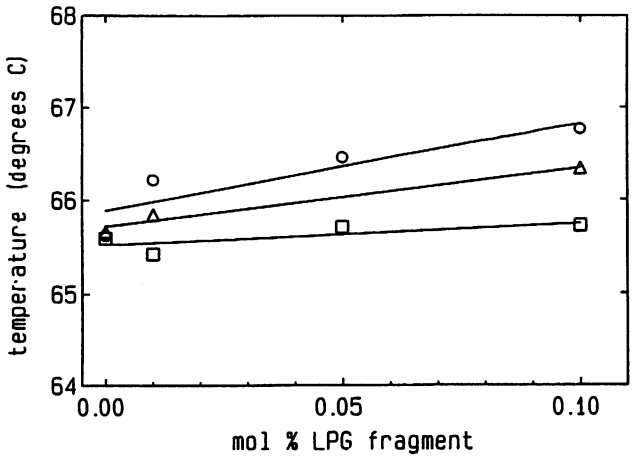

FIG. 5. Effect of LPG fragments on the bilayer to hexagonal phase transition temperature of DEPE multilamellar vesicles. LPG fragments were of 1 (□), 6 (△), or 12 (○), repeating disaccharide units. T_H is plotted against mol% LPG fragment added to the MLVs.

since the percentage bound is larger than the percentage that is active, similar to the effects seen in Fig. 2 containing LPG on the outer monolayer only. The effects of LPG sidedness on PKC_α activity are similar toward both histone and the MARCKS peptide again suggesting that the LPG effects are independent of substrate availability (not shown).

We have also tested the inhibition of PKC by LPG contained on the opposing monolayer. This was done by incorporating LPG on both sides of the membrane followed by extraction of LPG on the outer monolayer with MLVs. As a control, we also extracted LPG from LUVs containing the amphiphile only on the outer monolayer. In this latter case, all of the LPG should be extracted and these LUVs are capable of supporting close to 80% of the total activity (Fig. 7). For LUVs originally containing LPG on both sides of the membrane, extraction with MLVs resulted is vesicles supporting a substantially lower activity, particularly in the case of vesicles originally containing 5% LPG (Fig. 7). This result demonstrates that LPG on the inner monolayer can inhibit PKC bound to the outer monolayer. However, this transbilayer inhibition is weaker than in

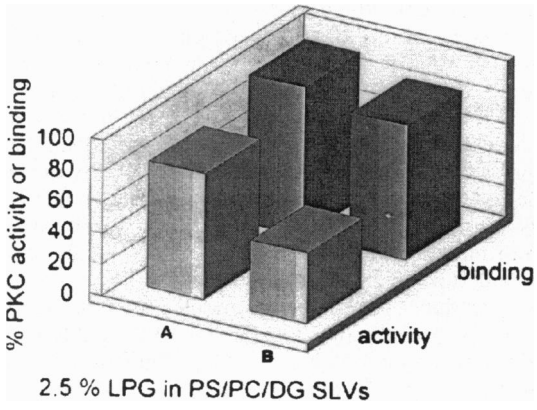

FIG. 6. Effect of LPG added to one vs. both sides of the membrane on the activity of PKC_α toward histone as compared with its binding to SLVs. Vesicles were composed of 30 mol% POPS, 1 mol% DG, and 2.5 mol% LPG (with respect to total lipid) added to the outside (A) or to each side (B) (5% LPG of total lipid) of the membrane, and the remainder POPC. SD (not shown) did not exceed ± 7%. Binding and activity are expressed as a percentage of that obtained with vesicles containing no LPG. This corresponds to 100% of the total added enzyme bound, with a specific activity of 3.3 nmol·min·mg. Data are expressed as the mean of triplicate determinations.

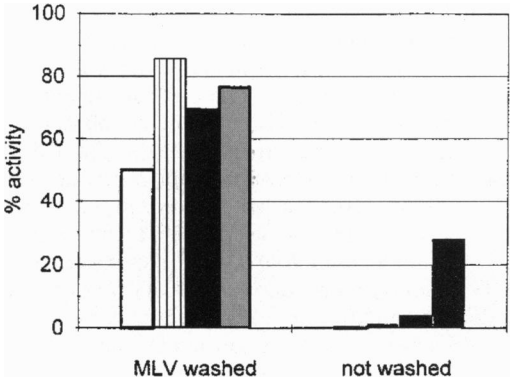

FIG. 7. LPG on the inner monolayer of LUVs inhibits the activity of PKC toward histone at the outer monolayer. Vesicles were composed of 20 mol% POPS, 1 mol% DG, 2.5 or 5 mol% LPG initially added to both monolayers, and the remainder POPC. Vesicles were incubated with or without sucrose-loaded MLVs for 48 hr. MLVs were sedimented, and the remaining supernatant contained LUVs with LPG removed from the outer monolayer. These LUVs were used in PKC activity assays. The left-hand group of bars represent activity using LUVs that had been washed with MLVs and include: LPG originally on both sides of the bilayer at 5% □ or at 2.5% ■ total lipid and finally containing 2.5% □ or 1.25% ■ LPG on the inner monolayer only, after the wash. Controls originally containing 5% (▥) or 2.5% (▩) LPG, originally on the outer monolayer and finally having almost all of the LPG removed by the MLV wash. The right-hand group of bars correspond to the original LUVs described above and have not had any LPG extracted with MLVs. SD (not shown) did not exceed ± 8%. Activity was expressed as a percentage of that obtained with vesicles that did not have LPG incorporated. Data are expressed as the mean of triplicate determinations.

cases where LPG is present in the outer monolayer or in both monolayers (no MLV wash, right side of Fig. 7).

DISCUSSION

The inhibition of the PKC of host cells by LPG may have a biological role for the survival of *Leishmania* in macrophages. *Leishmania* is one of only a few microbes that can successfully survive in lysosomes of host macrophages. LPG has been shown to be critical in the infection of a macrophage since *Leishmania* mutants that lack LPG are unable to survive (1). Moreover, the addition of purified LPG to these mutants prolonged their capability of intracellular survival in macrophages. Furthermore, preliminary work in our laboratories has shown that genetic complementation of these mutants restores the expression of cell surface LPG and their ability to infect macrophages, thereby demonstrating the importance of LPG in *Leishmania*-macrophage interactions. The underlying basis for surviving with impunity is accomplished at least in part, by preventing macrophages from becoming activated, a process mediated by PKC.

LPG represents a modulator of PKC activity that is structurally different from those previously studied. A knowledge of its mechanism of inhibition of this enzyme may contribute to a further understanding of the modulation of PKC activity by changes in membrane properties. An interesting finding was that LPG could inhibit both membrane binding and the activity of membrane-bound PKC_α from the opposite monolayer. The effects of LPG from the inner monolayer of LUVs on PKCs binding and activity are not quite as strong as its effects from the outer monolayer [compare effects of 5 mol% LPG added to the outer monolayer on binding (Fig. 2*a*) and activity (Fig. 2*b*) of PKC at 30 mol% PS with the effects of 2.5 mol% LPG added to both inner and outer monolayers on binding and activity (Fig. 6)]. However, there is still a significant amount of inhibition, which supports the hypothesis that the inhibition of

PKC by LPG is (at least partially) a consequence of the modulation of membrane biophysical properties. Similar results were obtained when the LPG was removed from the outer monolayer and left only on the inner monolayer. Compare differences in % activity when 5% LPG was originally present on the outer monolayer and then removed with MLVs, with the activity when LPG was originally present on both monolayers (Fig. 7). In the later case 2.5% LPG remains on the inner monolayer and can cause inhibition of PKC activity on the outer monolayer. This provides stronger evidence that LPG can affect PKC activity from the opposite monolayer.

It has been suggested that modulation of PKC activity by additives to the membrane is predictable on the basis of the charge on the additive and its effect on lipid polymorphism (23). In general, cationic substances are inhibitors of PKC, while anionic membrane additives are either activators or cofactors for the enzyme. In addition, uncharged or zwitterionic additives that raise the T_H of the membrane are inhibitors of PKC. LPG is very potent in raising T_H (20); however, LPG is also anionic. Generally the charge effect predominates for modulation of PKC activity, but not in the case of LPG. LPG may be the only known anionic amphiphile inhibitor of PKC. This may be a consequence of the unusually large effect of LPG in raising T_H, which is a property correlated with the inhibition of PKC.

The activity of PKC toward histone is modulated by the calcium concentration at 0.3 nm from the membrane surface (24). Under the conditions of our experiment, however, LPG does not modulate PKC activity solely by altering the amount or distribution of Ca^{2+} at the membrane surface since we find that the effect of LPG on inhibiting the phosphorylation of histone is independent of Ca^{2+} concentration between 0–10 mM calcium (results not shown).

Kinetic analysis indicates that LPG affects K_m of the MARCKS peptide but not the V_{max}. In addition, analysis of susceptibility to tryptic cleavage indicates that PKC bound to the membrane in the presence of LPG does not undergo a conformational change associated with activation of the enzyme, as occurs in the absence of LPG. These results indicate that the active site of PKC is less available to substrate when the enzyme is bound to membranes in the presence of LPG. However, the native structure and catalytic mechanism of PKC remain unaltered by LPG as indicated by the invariance of V_{max}. This is also shown by the fact that the PKC-catalyzed phosphorylation of protamine sulfate is not inhibited by LPG.

This is the first example of an amphiphile that inhibits the membrane-bound form of PKC. PKC activity is generally well correlated with membrane binding except for the LPG-containing membranes, which are unique in strongly inhibiting the membrane-bound form of the enzyme. How LPG accomplishes this is still uncertain. It is possible that LPG may alter the site at which PKC binds to the membrane by binding to the enzyme itself. A number of anionic amphiphiles of varying chemical structure can bind to PKC (14, 26). In addition, substances that raise T_H appear to make the rearrangement of proteins in membranes more difficult. For example, this has been suggested as a factor modulating the lifetime of alamethicin channels (27) as well as the bilayer disrupting effects of viral fusion proteins (20), and the inhibition of insulin receptor autophosphorylation and signaling (28).

Thus, LPG can function both to prevent binding of PKC to membranes as well as to inhibit the membrane-bound form of this enzyme. This is the first time that an amphiphile has been shown to inhibit the membrane-bound form of PKC in the presence of other cofactors. Our findings also demonstrate the ability of membrane modulation of protein activity to be transmitted by the coupling of opposing monolayers. This mechanism may be of importance to the survival of *L. donovani*, and may therefore be a target in the control of the spread of the parasite.

We thank Mr. Robert Bruins for his help in purifying PKC_α and Drs. D. Burns and N. Rankl for providing the cell pellets used in this purification. This work was supported by Medical Research Council of Canada Grant MA-7654 to R.M.E. and by National Institutes of Health Grant AI20941 to S.J.T.

1. Turco, S. J. & Descoteaux, A. (1992) *Annu. Rev. Microbiol.* **46,** 65–94.
2. McConville, M. J. & Ferguson, M. A. J. (1993) *Biochem. J.* **294,** 305–324.
3. Wilson, E., Olcott, M. C., Bell, R., Merrill, M., Jr., & Lambeth, J. D. (1986) *J. Biol. Chem.* **261,** 12616–12623.
4. Descoteaux, A. & Turco, S. J. (1993) *Parasitol. Today* **9,** 468–471.
5. Descoteaux, A., Matlashewski, G. & Turco, S. J. (1992) *J. Immunol.* **149,** 3008–3015.
6. Descoteaux, A., Turco, S. J., Sacks, D. L. & Matlashewski, G. (1991) *J. Immunol.* **146,** 2747–2753.
7. McNeely, T. B. & Turco, S. J. (1990) *J. Immunol.* **144,** 2745–2750.
8. McNeely, T. B., Rosen, G., Londner, M. V. & Turco, S. J. (1989) *Biochem. J.* **250,** 601–604.
9. McNeely, T. B. & Turco, S. J. (1987) *Biochem. Biophys. Res. Commun.* **148,** 653–657.
10. Tolson, D. L., Turco, S. J. & Pearson, T. W. (1990) *Infect. Immun.* **58,** 3500–3507.
11. Orlandi, P. A., Jr., & Turco, S. J. (1987) *J. Biol. Chem.* **262,** 10384–10391.
12. Turco, S. J., Wilkerson, M. A. & Clawson, D. R. (1984) *J. Biol. Chem.* **259,** 3883–3889.
13. Dubois, M., Grilss, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28,** 350–356.
14. Huang, K.-P., Chan, K. F., Singh, T. J., Nakabayashi, H. & Huang, F. L. (1986) *J. Biol. Chem.* **261,** 12134–12140.
15. Mosior, M. & Epand, R. M. (1993) *Biochemistry* **32,** 66–74.
16. Hannun, Y. A., Loomis, C. R. & Bell, R. M. (1985) *J. Biol. Chem.* **260,** 10039–10043.
17. Stabel, S., Schaap, D. & Parker, P. J. (1991) *Methods Enzymol.* **200,** 670–763.
18. Rebecchi, M., Peterson, A. & McLaughlin, S. (1992) *Biochemistry* **31,** 12742–12747.
19. Kaibuchi, K., Takai, Y. & Nishizuka, Y. (1981) *J. Biol. Chem.* **256,** 7146–7149.
20. Ling, M., Stafford, A., Nir, S., Turco, S. J., Flanagan, T. D. & Epand, R. M. (1995) *Biochemistry* **34,** 4676–4683.
21. Newton, A. C. & Koshland, D. E., Jr. (1989) *J. Biol. Chem.* **264,** 14909–14915.
22. Ferrari, S., Marchiori, F., Marin, O. & Pinna, L. A. (1987) *Eur. J. Biochem.* **163,** 481–487.
23. Epand, R. M. (1992) in *Protein Kianse C: Current Concepts and Future Perspectives,* eds. Lester, D. S. & Epand, R. M. (Ellis Horwood, Chichester, U.K.), pp. 135–156.
24. Mosior, M. & Epand, R. M. (1994) *J. Biol. Chem.* **269,** 13798–13805.
25. Bazzi, M. D. & Nelsestuen, G. L. (1987) *Biochem. Biophys. Res. Commun.* **147,** 248–253.
26. Mosior, M., Golini, E. S. & Epand, R. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 1907–1912.
27. Keller, S. L., Bezrukov, S. M., Gruner, S. M., Tate, M. W., Vodyanoy, I. & Parsegian, V. A. (1963) *Biophys. J.* **65,** 23–27.
28. McCallum, C. D. & Epand, R. M. (1995) *Biochemistry* **34,** 1815–1824.